Table S1: Crystallographic Data and Refinement Statistics (Related to Figure 2 and Figure 6)

Data Collection	**B30.2**	**B30.2/cHDMAPP Complex**
Space group	$P22_12_1$	$P22_12_1$
Cell dimensions		
a, *b*, *c* (Å)	39.03, 44.95, 124.81	38.98, 44.95, 125.26
α, β, γ (°)	90, 90, 90	90, 90, 90
Resolution (Å)	21.0-1.40 (1.45-1.40)	37.2-1.46 (1.51-1.46)
R_{merge}	0.069 (0.320)	0.037 (0.482)
$I/\sigma I$	27.1 (6.5)	16.7 (2.0)
Completeness (%)	96.2 (90.2)	95.8 (85.1)
Redundancy	5.3 (5.4)	2.9 (2.8)
Refinement		
Resolution (Å)	21.0-1.4	37.2-1.46
No. reflections	42350	37505
R_{work}/R_{free}	13.51/16.64	15.33/18.96
No. atoms		
Protein	1563	1535
Ligands	22	21
Water	203	211
B-factors		
Macromolecules	11.7	18.0
Water	28.6	34.3
Rmsds		
Bond lengths (Å)	0.011	0.011
Bond angles (°)	1.06	1.09
Ramachandran		
Most Favored (%)	91.2	87.5
Additionally Allowed	8.8	12.5
Generously Allowed	0.0	0.0
Disallowed	0.0	0.0

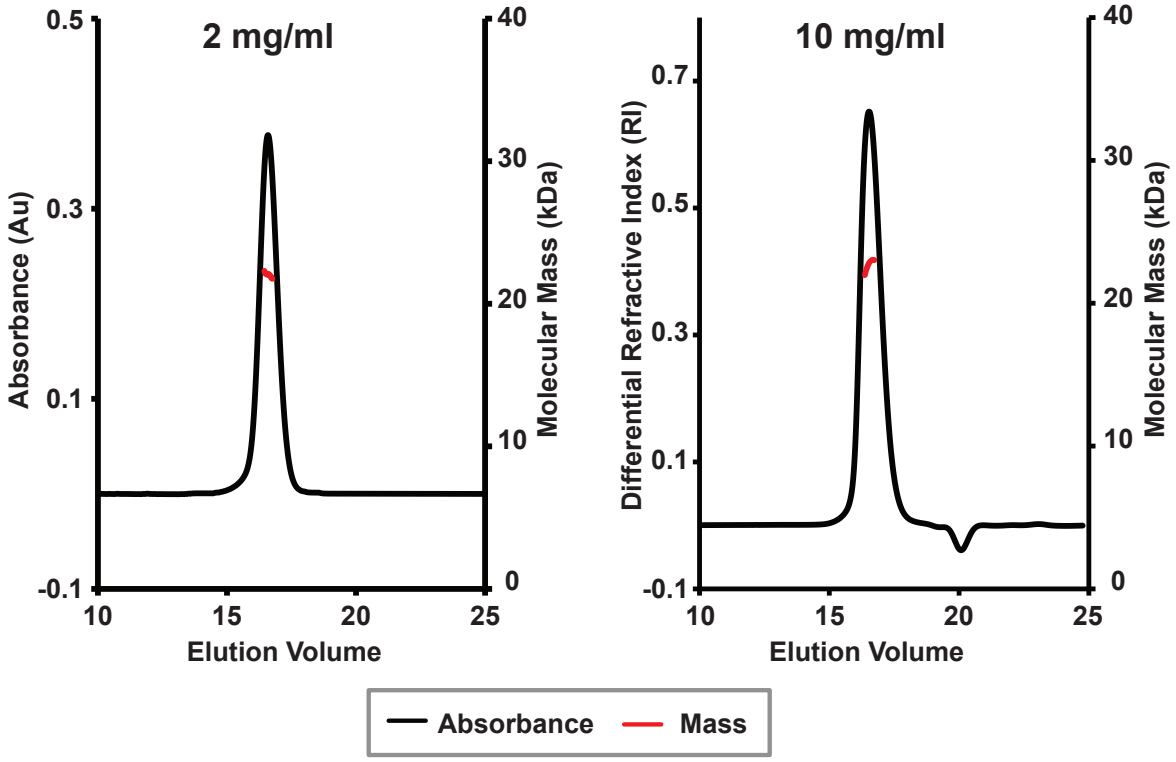

Figure S1. MALS analysis of BTN3A1 B30.2 Domain (Related to Figure 2): MALS analysis of BTN3A1 B30.2 domain. 500µl of B30.2 at 2mg/ml (left) or 250µl at 10mg/ml (right) was injected over a Superdex 200 column and analyzed by MALS. B30.2 elutes as a single peak as measured by UV280 absorbance or differential refractive index (black). MALS calculation of the molecular weight (red) corresponds to that of the monomer: 22.020 ± 0.09 kD at 2 mg/ml and 22.700 ± 0.11 kD at 10mg/ml.

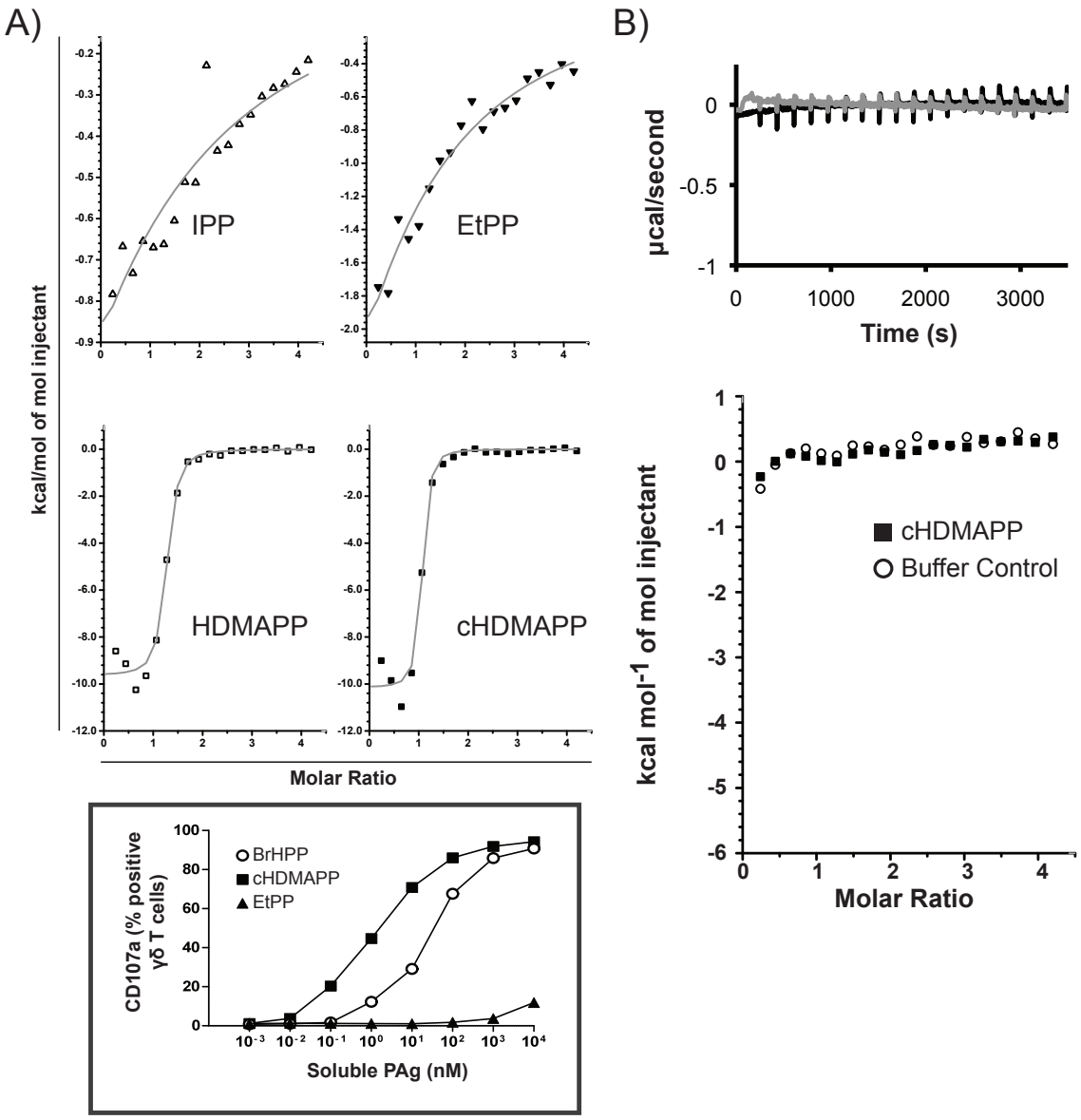

Figure S2 Fits of pAg binding to B30.2 and extracellular domain of BTN3A1 (Related to Figure 3): **A)** Top: Fitting of PAg binding to BTN3A1 B30.2 domain using ITC. ITC data for each of the phosphoantigens is shown as follows: IPP as open triangles, EtPP as filled triangles, HDMAPP as open squares, and cHDMAPP as filled squares. Fits for each binding isotherm are shown in gray. Bottom: Comparison of the functional potencies of soluble PAgs. CD107a expression on human Vγ9Vδ2 T cells induced by grading doses of soluble PAg (BrHPP, cHDMAPP and etPP) after a 5 hour incubation (autopresentation). The values for the percentage of CD107a+ γδ T cells are indicated on the graph. Similar results were obtained for IFN-γ and TNF-α release. **B)** Binding measurements of cHDMAPP to the extracellular domain of BTN3A1 (black) or buffer control (gray) at 15°C (top panel). Measurements were performed with 100µM BTN3A1 extracellular domain in the cell and 2mM cHDMAPP in the syringe. The binding isotherms are shown with buffer control (open circle) or cHDMAPP (filled square) (bottom panel).

Table S2: Thermodynamic binding parameters determined by ITC of various PAgs for the BTN3A1 B30.2 domain (Related to Figure 3).

	Stoichiometry (N)	K_D	ΔH (kcal/mol)	ΔS (cal/mol/deg)
IPP	1	0.49 ± 0.10 mM	-5.0 ± 0.7	-1.69
EtPP	0.90 ± 0.94	0.28 ± 0.15 mM	-8.1 ± 1.0	-10.8
HDMAPP	1.19 ± 0.017	0.92 ± 0.30 μM	-9.7 ± 0.2	-4.74
cHDMAPP	0.98 ± 0.015	0.51 ± 0.23 μM	-10.2 ± 0.3	-5.3

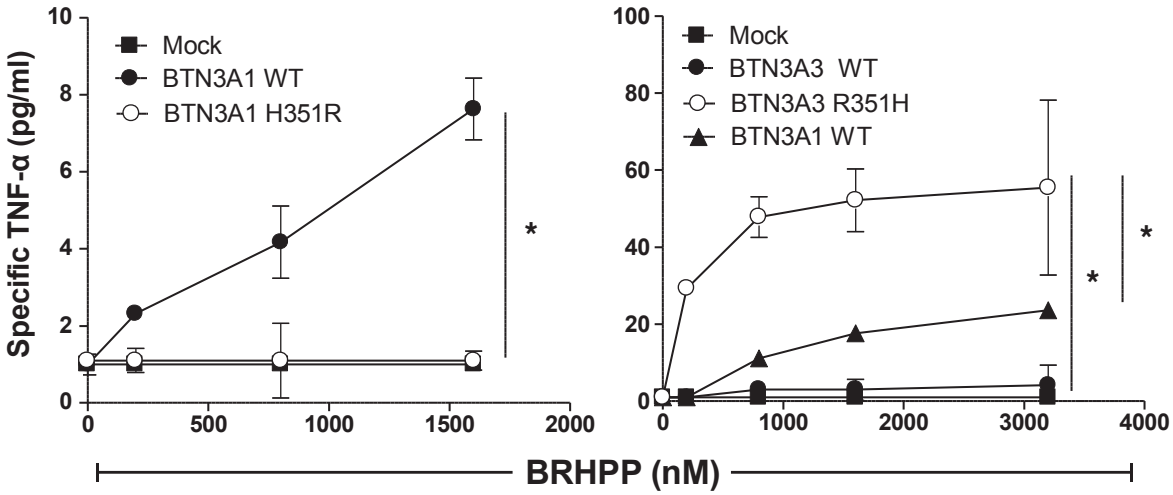

Figure S3. BrHPP treatment of BTN3A1 and BTN3A3 B30.2 domain mutants (Related to Figure 5): TNF-α release from Vγ9Vδ2 T cells induced by grading doses of soluble PAg (BrHPP) after a 6 hour co-culture with HEK sh 284 cells either mock-transfected (Mock) or expressing, 36 h after transient transfection, BTN3A1 WT or BTN3A1 H351R mutant (left panel), or expressing BTN3A3 WT or BTN3A3 R351H (right panel). Data are mean of duplicates ± SD and are representative of three independent experiments. * *P* <0.05 (paired Student *t*-tes*t*). TNF-α concentration was assessed by a biologic assay based on WEHI164 clone 13 cell viability. Effector to Target ratio: 1/10. The supernatants collected after co-coculture were diluted (1/10) prior addition on WEHI cells.

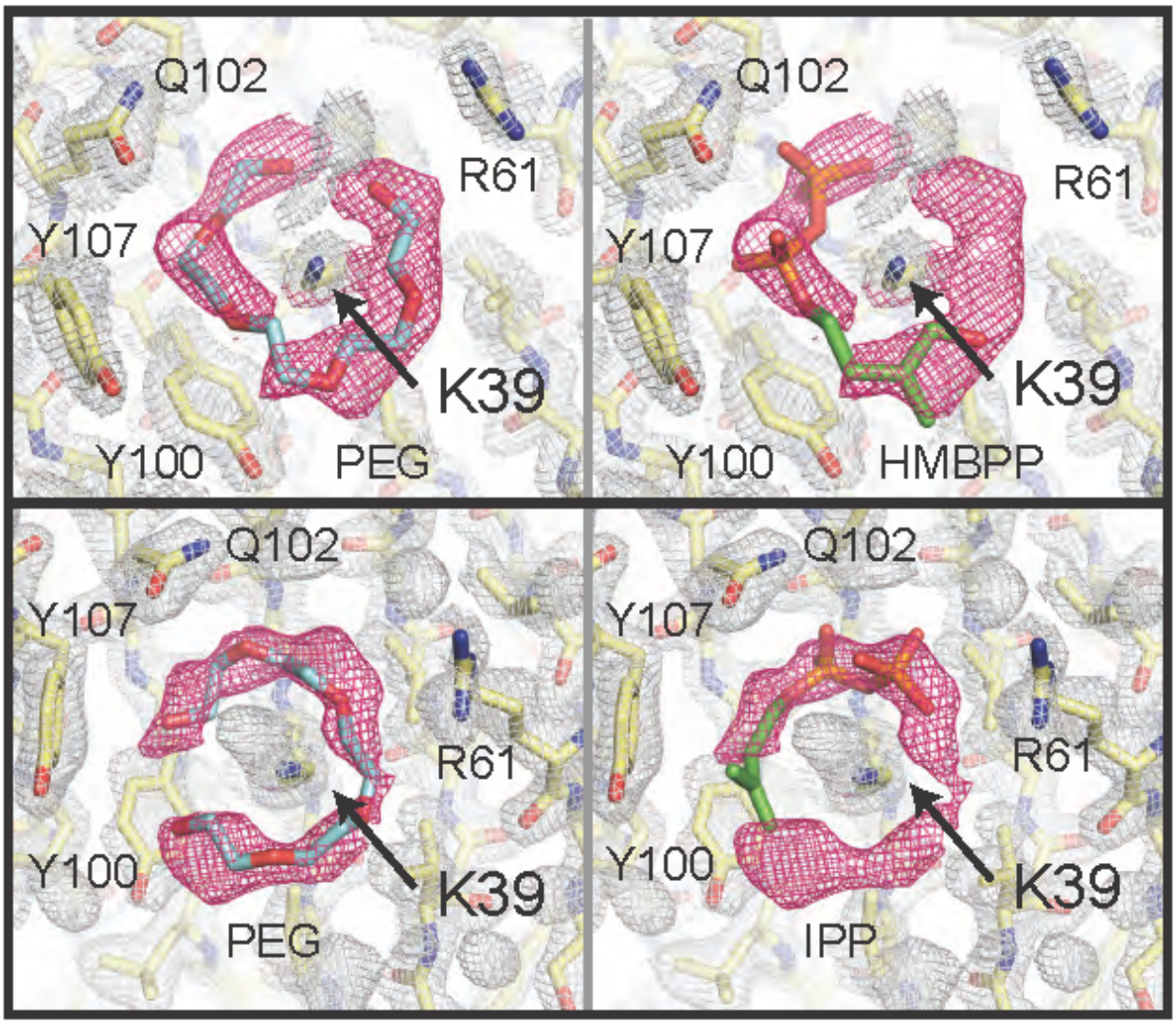

Figure S4. Omit map electron density of BTN3A_1-phosphoantigen complexes. (Related to Figure 6): Omit map electron density of BTN3A_1/phosphoantigen complexes. Omit maps with ligand removed were calculated using the models and structure factors from PDB IDs: 4K55 (HMBPP/BTN3A_1) on top and 4JKW (IPP/BTN3A_1) at bottom. Electron density is contoured at 1σ and shown in hot pink for ligand and white for BTN3A. PEG molecules are modeled into the maps at left and are shown in cyan (carbon) and red (oxygen). Right panels show the positioning of the respective phosphoantigens in the respective PDB coordinate files.

Supplemental Experimental Procedures (Related to Experimental Procedures):

Expression of full-length and chimeric BTN3 proteins into rodent cells.

Full-length cDNAs for human BTN3A1 (LIFESEQ3294566), and BTN3A3 (BC015815) were obtained from Open Biosystems. cDNAs silently mutated in the sh284 target sequence were subcloned in a modified pIRES1hyg vector (Clontech) as already described (Harly et al., 2012). Plasmids were used for transient or stable transfections in either BTN3A silenced HEK293FT cells (sh#284; clone#30) or wild-type HEK293 cells. Plasmids encoding for chimeric BTN3A1 and BTN3A3 carrying swapped, truncated or mutated B30.2 domains were generated by cloning or PCR according to standard procedures. Transient transfections of human (HEK293; HEK sh284) and rodent cells were done using Lipofectamine 2000 (Invitrogen/LifeTechnologies) according to the supplier's instruction. Human ICAM-1 cDNA was cloned into the pCIneo vector (Promega) and was kindly provided by Dr A. Moreau-Aubry (INSERM UMR892, Nantes, France). ICAM-1 plasmid was co-transfected (ratio 1/1) with human BNT3A1 or A2 plasmids for transfection into rodent cells. Following transient transfection, HEK transfectants were checked for the selective re-expression of mutated BTN3A1 or BTN3A3 by flow cytometry and expression of human BTN3A and ICAM-1 was checked by flow cytometry after transfection into rodent cell lines.

Expression of full-length chimeric BTN3A1 proteins for FRAP analysis.

Carboxy-terminus mCherry-fused BTN3A1 molecules were obtained by subcloning full-length wildtype or H351R mutated cDNAs in frame with the sequence encoding for the fluorescent tag from pmCherry-N1 vector (Clontech). Helix-forming peptide linker A(EAAAK)4A was introduced in frame to separate BTN3A1 and fluorescent domains. Plasmids were transiently or stably transfected in BTN3A silenced HEK293FT cells (sh#284; clone#30) or HEK293 cells, for functional or FRAP assays, respectively. The expression of fluorescent BNT3A1 on HEK transfected cells was checked by flow cytometry and stable transfectants were sorted after selection using a FACS AriaIII cell sorter (BD Bioscience).

FRAP experiments and data analysis.

HEK293 cells stably expressing either wildtype or mutated B30.2 carboxy-terminus mCherry-fused BTN3A1 were laid on m-slides (Ibidi) and analyzed using a Nikon A1RS confocal microscope (60xNA 1.40 oil immersion objective). Selected rectangular areas were photobleached for 500 ms by using full power of laser intensity (> 90% of loss of fluorescence). Images were collected every 5 s, before (30 s) and after (120 s) bleaching using low laser intensity. The resulting curves were fitted using one-phase exponential equations.

Protein Expression and Purification

The BTN3A1 B30.2 domain and associated mutants were cloned into pET28a with a six-HIS tag and expressed in BL21 strain *Escherichia coli*. Cells were grown to OD_{600}=0.6 and then induced for four hours at room temperature. Protein was harvested and purified using Ni-NTA (Qiagen) IMAC chromatography in 20mM Tris pH8.0, 400mM NaCL, 20mM Imidazole, eluted with 20mM Tris pH8.0, 400mM NaCL, 250mM Imidazole, and desalted into 10mM Hepes pH7.2, 150mM NaCl, 0.02% azide using an Econo-Pac 10DG column (Biorad). Protein was cleaved overnight with carboxypeptidase A (Sigma) at room temperature. Protein was further purified by gel filtration over a Superdex 200 column (GE healthcare) in 10mM Hepes pH7.2, 150mM NaCl, 0.02% azide). Protein concentration was initially determined by BCA and matched A_{280} measurements using the theoretical extinction coefficient. BTN3A1 extracellular domain and the 20.1 single chain antibody were expressed and purified as previously described (Palakodeti et al., 2012). The G115 Vγ9Vδ2 TCR γ and δ chains were separately cloned into different versions of the pAcGP67A vector each containing a 3C protease site followed by either acidic or basic zippers and a 6xHis tag. Additionally, a BirA biotinylation sequence was engineered following the δ chain but preceding the 3C site. Proteins were co-expressed in Hi-5 cells using baculovirus transduction. Proteins were initially purified using Ni-NTA IMAC chromatography and cleaved using 3C protease. Cleaved samples were then biotinylated using the BirA enzyme and purified using size exclusion chromatography over a Superdex200 column as above. The Vγ4Vδ1 and G8 TCRs and T22 were cloned and expressed as previously described (Adams et al., 2005; Luoma et al., 2013).

MALS Analysis of BTN3A1 B30.2 domain

500µl of BTN3A1 B30.2 domain at 2mg/ml or 250µl at 10mg/ml protein concentration was injected over a Superdex 200 column with an in line Dawn Heleos MALS detector (Wyatt) and Optilab Trex refractive index detector (Wyatt) in 10mM Hepes pH7.2, 150mM NaCl, 0.02% azide at 25°C. Protein elution was measure using the UV280 absorbance for the 2mg/ml peak and through the differential refractive index for the 10mg/ml peak, as the sample concentration was too high to analyze by UV280. Average particle size was determined using Astra software (Wyatt) fit over the major peak.

Surface Plasmon Resonance

Purified and biotinylated G115 TCR was immobilized on a streptavidin coated chip (Sensor Chip SA, GE Healthcare) to a total signal of 6500RU. BTN3A1 extracellular domain, 20.1 single chain antibody, and BTN3A1 20.1 complex were purified in SPR running buffer (10mM Hepes pH7.4, 150mM NaCL, and 0.05% Tween20) using gel filtration on a Superdex 200 column. Peak fractions were diluted to 4µM and serially diluted to the following concentrations: 2µM, 1µM, 0.5µM, 0.25µM, 0.125µM, 0.0625µM, and 0.03125µM. SPR measurements were collected on a Biacore2000 at 25°C. Streptavidin conjugated AmDex polymers were obtained from Fina Biosolutions. 60ug of

biotinylated TCRs were mixed with 1μg AmDex to create multimerized TCRs. Biotinylated BTN3A1 or T22 were immobilized as above saturating at 3000RU. AmDex conjugates were injected at the following AmDex concentrations: 0.03125μg/ml, 0.0625μg/ml, 0.125μg/ml, 0.25μg/ml, 0.5μg/ml, and 1μg/ml. Due to non-specific interactions with the biotin blocked reference flowcell binding was measure as the difference between the observed binding to BTN3A1 and T22 for G115 and the Vγ4Vδ1 TCR. The binding of the G8 TCR was determined by subtracting the BTN3A1 signal from the T22 signal.

SPR K_D estimation

The maximal detectable KD estimation was roughly approximated using basic equilibrium binding: %bound = [ligand]/(KD + [ligand]). Assuming a KD of 500mM and using the maximum ligand (BTN3A1) concentration of 4mM, ~0.8% of the ligand should be bound to the surface during flow. ~6500RU of protein was immobilized on the surface of the chip in a functionally relevant orientation (site-specific biotinylated the C terminus of the TCR immobilized on a streptavidin sensor chip) an estimated binding affinity of 500mM should give rise to a signal of approximately 50RU.

Supplemental References:

Adams, E.J., Chien, Y.H., and Garcia, K.C. (2005). Structure of a gammadelta T cell receptor in complex with the nonclassical MHC T22. Science *308*, 227-231.

Harly, C., Guillaume, Y., Nedellec, S., Peigne, C.M., Monkkonen, H., Monkkonen, J., Li, J., Kuball, J., Adams, E.J., Netzer, S.*, et al.* (2012). Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human gammadelta T-cell subset. Blood *120*, 2269-2279.

Luoma, A.M., Castro, C.D., Mayassi, T., Bembinster, L.A., Bai, L., Picard, D., Anderson, B., Scharf, L., Kung, J.E., Sibener, L.V.*, et al.* (2013). Crystal Structure of Vdelta1 T Cell Receptor in Complex with CD1d-Sulfatide Shows MHC-like Recognition of a Self-Lipid by Human gammadelta T Cells. Immunity *39*, 1032-1042.

Palakodeti, A., Sandstrom, A., Sundaresan, L., Harly, C., Nedellec, S., Olive, D., Scotet, E., Bonneville, M., and Adams, E.J. (2012). The molecular basis for modulation of human Vgamma9Vdelta2 T cell responses by CD277/butyrophilin-3 (BTN3A)-specific antibodies. J Biol Chem *287*, 32780-32790.